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TITLE: Pathogenesis of Ovarian Serous Carcinoma as the Basis
for Immunologic Directed Diagnosis and Treatment

SUBTITLE: Project 2 - Identification of Autologous Antigens
in Early Stage Serous Carcinoma

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13. ABSTRACT (Maximum 200 Words) Our goal is to develop an early detection screening test for serous carcinoma. Specific aim 1: Obtain cDNAs of autologous tumor antigens recognized by sera of patients with early stage serous carcinoma, but not controls. Progress: While our ovarian cancer cDNA library is construction for SEREX analysis, we have developed an alternative, complementary methodology. We employed patient sera to immunoprecipitate proteins from autologous cancer cells and identified these antigens by mass spectrometry. One antigen, SMAP-1, has homology to genes that facilitate cell division. Specific aim 2: Identify autologous tumor antigens expressed in serous carcinoma but absent from, or a low level in normal tissue. Progress: We have expressed recombinant SMAP-1 protein and generated specific antisera. We the SMAP-1 mRNA is ubiquitous in all tissues tested thus far, and immunohistochemical studies are currently underway. We have already several identified several other SEREX antigens, including the homeobox HOXB7 transcription factor. We have generated peptide antiserum to HOXB7 and confirmed its over expression in cancer cell lines. Interestingly, we also note a shift from a nuclear localization in normal tissue to the cytoplasm in most carcinomas.				
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Introduction

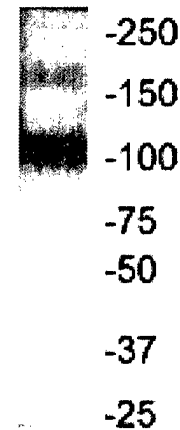
The immune system constantly surveys the body for 'non-self' antigens, and generates a response in the appropriate context. A key finding of cancer biology is that cancer patients often generate antibody to neoantigens specifically expressed in their tumor (1). Autologous antibodies have been documented in patients afflicted with a variety of different cancers, (2-4) including ovarian cancer (5). Autologous antibodies generated by cancer patients have been used to screen expression libraries for tumor antigens. This technique, originally described by Sahin *et al* and termed SEREX (serologic analysis of recombinant cDNA expression libraries of human tumors with autologous serum), has been used to obtain tumor antigen cDNA clones. SEREX has been applied to tumors of many organs (6) and antibody specific for antigens identified by SEREX in other cancer types have been demonstrated in ovarian cancer patients (7, 8). Tumor-specific autoantigens that are common among ovarian cancer patients but not recognized by sera of healthy volunteers have been identified by us e.g. HOXA7 and HOXB7, and others e.g. cathepsin D and GRP78 (9). Although expressed, Cathepsin D and GRP78 derived from normal tissue were not recognized by sera from ovarian cancer patients implying that they contain tumor-specific epitopes (9). Detection of these autologous antibody responses to ovarian cancer antigens appears to have prognostic significance (10). SEREX antigens derived from early stage serous carcinoma may represent useful biomarkers for the dissection of molecular pathways of serous carcinoma (Dr Shih, Project 1). SEREX antigens are also potential targets for cancer immunotherapy (11) (Dr Wu, Project 3).

Body

Objective 1: Obtain cDNAs of autologous tumor antigens recognized by sera of patients with early stage serous carcinoma, but not controls.

Task 1.1. Screen sera of patients (n=12) with early stage serous carcinoma by Western blot analysis to identify those patients with high titer autologous tumor-reactive antibody (months 0-2). We have performed Western blot analysis to select sera suitable for immunoscreening of a cDNA expression library. We have screened with sera from patients with micropapillary serous carcinoma, which has been proposed as a precursor/intermediate of serous carcinoma. We also tested immunoprecipitation, rather than Western blot, as a method to identify autologous tumor antigens. We derived a cell line from a micropapillary serous carcinoma of patient JH514. Affinity purified serum immunoglobulin from the same patient was covalently coupled to Sepharose beads, and these beads used to immunoprecipitate antigens from detergent lysate of the autologous JH514 cell line. The serum antibody from this patient recognized two autologous tumor associated antigens of approximately 100kDa and 200kDa (Fig. 1).

Figure 1. Immunoaffinity purification of autologous tumor antigens for micropapillary serous carcinoma of the ovary. Serum from a patient with MPSC, JH514, was passed over a protein-G spin column. After washing, the antibody was covalently linked with a cross-linker. The column was then washed with low pH elution buffer followed by PBS. A detergent lysate was prepared from a MPSC cell line derived from the same patient. This lysate was passed through the column. After washing, the column was eluted in low pH. The eluate was separated by SDS-PAGE on an 8% gel. The proteins were stained with Coomassie Blue. Antigens of ~100kDa and ~200kDa were excised for mass spectrometry.



Task 1.2. Generate serous carcinoma cDNA expression libraries and immuno-screen with autologous patient (n=3) serum antibody to identify SEREX antigens (months 2-14). The generation of a cDNA expression library derived from the serous carcinoma cell line OVCAR-3 is ongoing. During the first year of funding we have also developed a complementary technology for the identification of autologous tumor antigens that is based upon immunoprecipitation and antigen identification using mass spectrometry. This approach has a key advantage over SEREX screening in that the antigen is expressed in its native conformation and with the appropriate post-translational modifications. Therefore we excised the bands from the gel shown in Figure 1, subjected the antigens to trypsin digest and determine the charge/mass ratio for the peptides present by mass spectrometry (Fig 2 and not shown).

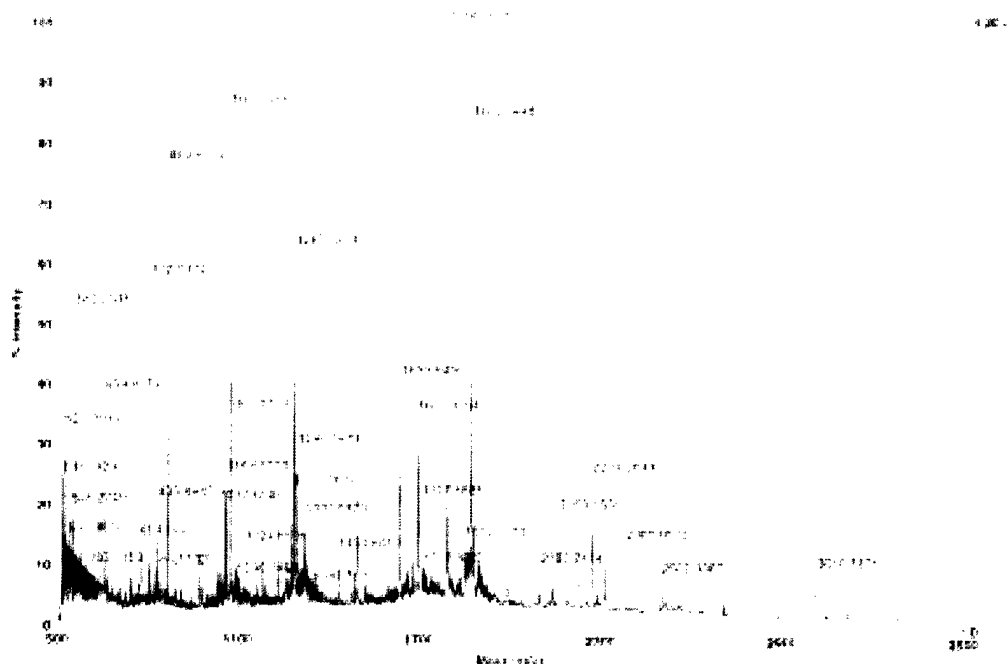


Figure 2. Mass spectrometry of tryptic peptides derived from the ~100kDa ovarian cancer-associated antigen.

Task 1.3. Sequence SEREX antigen cDNAs identified in screen and analyze sequences using BLAST searches (months 14-15).

The prospector website was used to search for proteins with matching peptides. The ~100kDa and ~200kDa antigens were identified by their peptide signatures as SMAP-1 (101kDa) (shown for SMAP-1 in Table 1) and KIAA1529 (195kDa). Nothing is known about the KIAA1529 antigen and no significant homologies or domains were identified. SMAP-1 has three TPR domains, and a recent report suggests that this protein is a chaperone that interacts with HSP90 and facilitates myosin motor assembly.

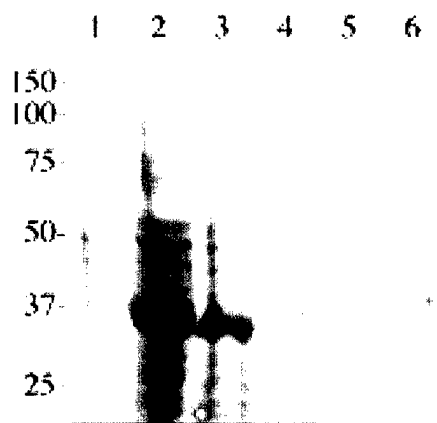
Table 1. Expected and observed M/z for peptides of SMAP-1 and the 100kDa antigen. Matched peptides cover 12% of the SMAP-1 protein, with 53 unmatched masses. 13/66 peptides match SMAP-1(AB014729), estimated MW of 101676.3Da, and SMAP-1b(AB014736).

M/z submitted	MH+ matched	Δ Da	Missed cleavages	Database Sequence
523.2691	523.2186	0.050	0	(K)CSEK(D)
562.2315	562.3201	-0.089	1	(K)GTEKK(Q)
1074.6897	1074.5618	0.13	1	(R)CVSLEPKNK(V)
1179.6649	1179.5618	0.085	0	(K)SWFEGQGLAGK(L)
1186.7039	1186.5414	0.16	0/1Met-ox	(K)LGSAGGTDFSMK(Q)
1287.7267	1287.7749	-0.048	1	(R)TVATLSILGTRR(V)
1320.6865	1320.7527	-0.066	0	(R)ASFITANGVSLK(D)
1661.9771	1661.8606	0.12	0	(R)LLDMGETDLMLAALR(T)
1677.9307	1677.8556	0.075	0/1Met-ox	(R)LLDMGETDLMLAALR(T)
1693.9454	1693.8505	0.095	0/2Met-ox	(R)LLDMGETDLMLAALR(T)
1797.9774	1797.9063	0.071	0	(R)WAVEGLAYLTFDADV(K)
2225.2600	2225.1119	0.15	0	(R)EIASTLMESEMMEILSVLAK(G)
2273.2726	2273.0966	0.18	0/3Met-ox	(R)EIASTLMESEMMEILSVLAK(G)

Task 1.4 Express SEREX antigens and purify from bacteria (months 15-21).

We obtained the full length cDNAs for human (MGC-999) and mouse SMAP-1 (MGC-7875). We expressed full length human SMAP-1 with a 6His tag in bacteria and purified the protein. Similarly, we expressed a central conserved region of murine SMAP-1 with a 6His tag and purified this protein from bacteria (Fig 3).

Figure 3. Purification of recombinant SMAP-1 conserved peptide from E.coli. A central portion of murine SMAP-1 was subcloned into pProExHT. Transformed E.coli DH5 α were induced for 6h in 1mM IPTG. Affinity purification on a Ni-NTA Sepharose column was performed according to Qiagen's protocol. Fractions were separated in a 12% SDS-PAGE gel and stained with Coomassie Blue. Lane 1: Uninduced E.coli, Lane 2: Induced E.coli, Lane 3: Lysate of induced E.coli, Lane 4: Column flow through, Lanes 5 and 6: Elution fraction.



Task 1.5 Determine the prevalence of serum antibody specific to each SEREX antigen by direct ELISA in a case/control study (months 21-27). (100 ovarian carcinoma patient sera, 102 sera from patients with benign ovarian tumors and 200 control sera will be tested.) We tested sera from nearly 600 patients, including over 70 with serous carcinoma and 19 with micropapillary serous carcinoma for reactivity with full length human SMAP-1. The data (not shown) suggested that antibody specific to SMAP was limited to patient JH-514.

Objective 2: Select autologous tumor antigens expressed in early stage serous carcinoma but absent from, or a low level in normal tissue.

Task 2.1. Generate rat antiserum to SEREX antigens (months 27-29)
In addition SMAP-1, we had previously identified HOXB7 as an ovarian tumor associated antigen. We generated antiserum to a unique peptide within HOXB7, as well as to a C-terminal peptide of human SMAP-1 and the central conserved region of mouse SMAP-1.

Task 2.2. Affinity purify and validate specificity of antibodies (months 29-32).
We have affinity purified the peptide antisera and validated their specificity by Western blot. Human SMAP-1 was expressed in 293 cells as a fusion with GFP and shown to accumulate in the cytoplasm (Fig. 4).

The antisera to the C-terminal peptide specifically recognized a ~130kDa protein lysates of cells expressing GFP-SMAP and a 100kDa protein in cells expressing unmodified SMAP-1 (Fig. 5). The antibody to the HOXB7 peptide recognized a single 25kDa band in ovarian tissue lysates, consistent with the molecular mass of the wild type protein, as well as recombinant 6His HOXB7. The antibody did not recognize the closely related HOXA7, demonstrating specificity.

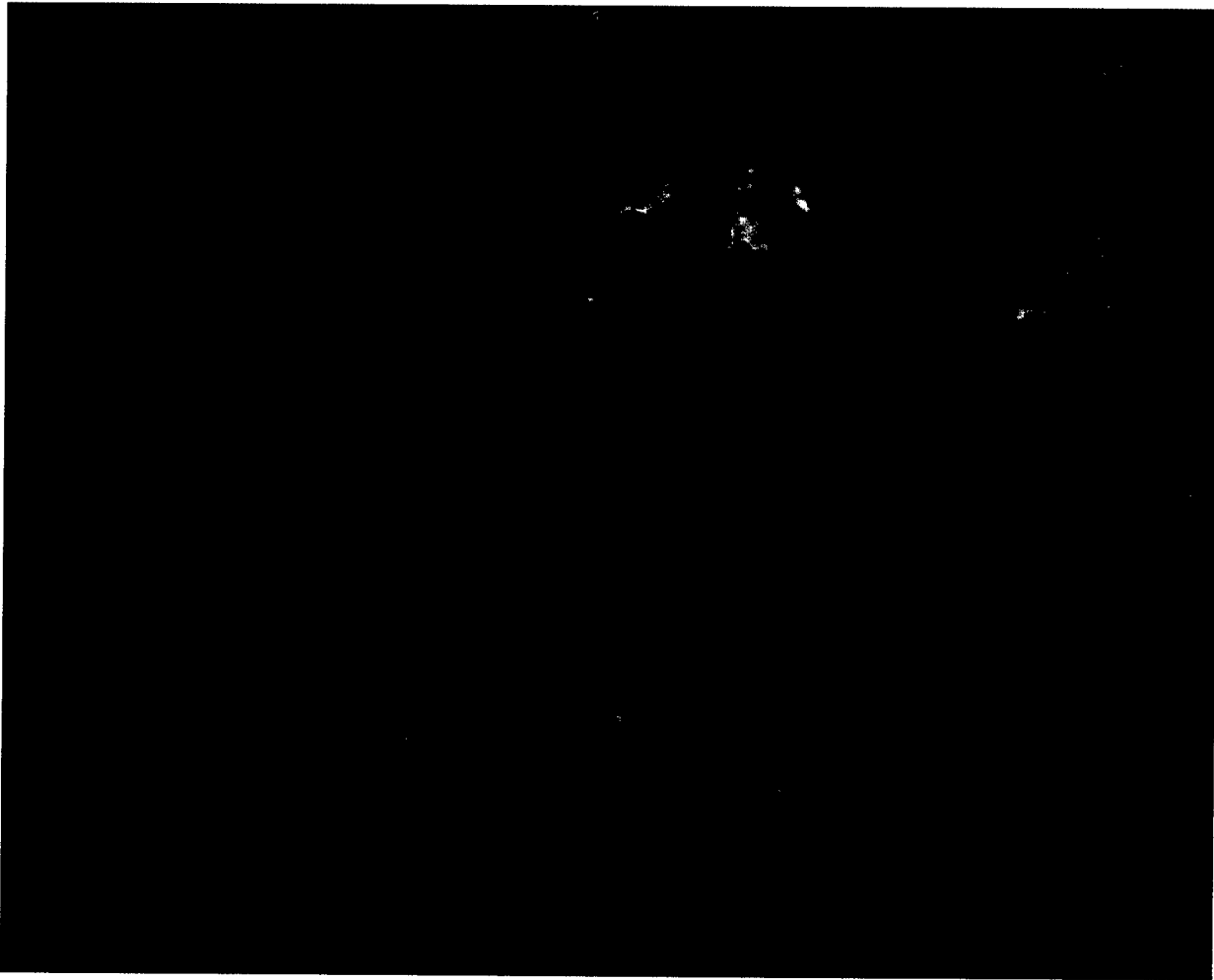
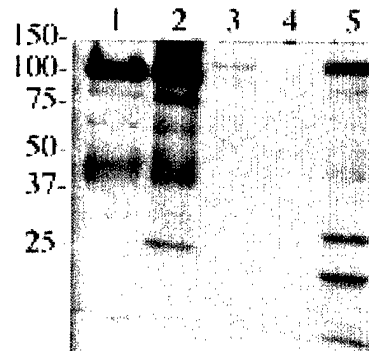


Figure 4. Subcellular localization of SMAP-1 fused to GFP. 293T cells were transfected with pEGFP-SMAP-1. At 72h after transfection, the cells were stained for DNA with DAPI (blue) and for actin with TRITC-phalloidin (red). The GFP-SMAP-1 (green) was visualized by confocal fluorescence microscopy.

Figure 5. SMAP-1-specific antibody. 6His-tagged recombinant SMAP-1 purified from E.coli (batches 1 and 2 in lanes 1 and 2) or lysates of SKOV3 (lanes 3 and 4) or 293T cells (lane 5) expressing either GFP-SMAP-1 (lanes 3 and 5) or GFP alone (lane 4) were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. Western blot analysis was performed using antiserum to the C-terminal 18 aa of human SMAP-1 at 1:1000 dilution and binding was detected by ECL.



Task 2.3. Examine the expression pattern of each SEREX antigen by immunohistochemical staining in normal ovary and a spectrum of ovarian tumors (months 32-36). (n>30 each for serous,

endometrioid, clear cell, mucinous and undifferentiated carcinoma of a range of stages, and $n > 30$ for serous borderline tumors, serous cystadenoma and normal ovary, giving an approximate total of 250 patient samples tested for each antigen).

We have used the HOXB7-specific antibody to immunostain tissue microarrays of ovarian tumors and normal human tissues.

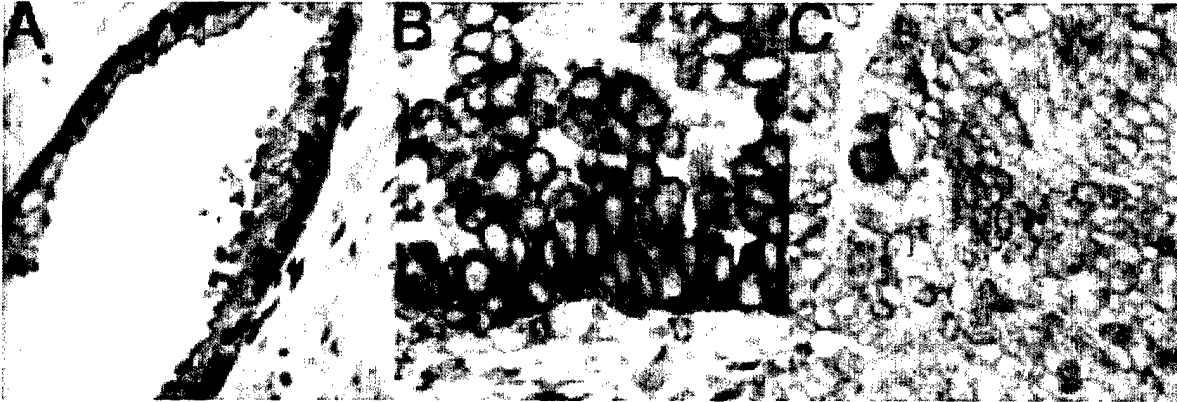


Figure 6. Immunohistochemical staining of HOXB7 in human tissues and tumors.

Tissue microarrays of normal human tissues and ovarian tumors were immunohistochemically stained using affinity purified antibody to a unique peptide within HOXB7. Panel A. Prostatic epithelium shows predominantly nuclear staining and the stromal is negative. Panels B and C: Cytoplasmic HOXB7 staining in two representative serous carcinomas of the ovary.

Our preliminary analysis suggests that HOXB7 is almost undetectable in normal ovarian surface epithelium, but is highly expressed in the majority of ovarian cancers. Interestingly, HOXB7 normally exhibits a nuclear localization, as expected for a transcription factor (see prostatic epithelium in Fig 6A). Excitingly, HOXB7 was mislocalized in the cytoplasm in the majority of ovarian serous carcinomas (examples shown in Figs 6B and C).

We also examined the expression of SMAP-1 transcripts in both normal tissues (Fig. 7) and in serous carcinoma of the ovary using Q-PCR (Fig. 8). SMAP-1 is widely expressed, but at notably higher levels in lung tissue. SMAP-1 transcripts were also detected at elevated levels in the majority of serous carcinoma tissues (including JH514 from which it was identified as a tumor antigen) as compared to levels in immortalized ovarian surface epithelial cells (IOSE-29). Immunohistochemical analysis of SMAP-1 expression in ovarian tumors and normal tissues will be completed in the near future.

Figure 7. Expression of murine SMAP-1 transcripts in normal tissues. Total RNA was extracted from fresh tissues or the cell line WF3. WF3 was derived by transformation of mesothelial cells with v-ras, HPV16 E6 and E7. WF3 provides an acitogenic murine model for ovarian cancer that is useful for tumor vaccine studies (see Dr Wu's project). After cDNA synthesis Q-PCR was performed for both actin and murine SMAP-1 (MGC-7875). Primers in neighboring exons of SMAP-1 were chosen. Single amplicons were determined by melting point analysis and gel electrophoresis. Controls lacking RT were negative.

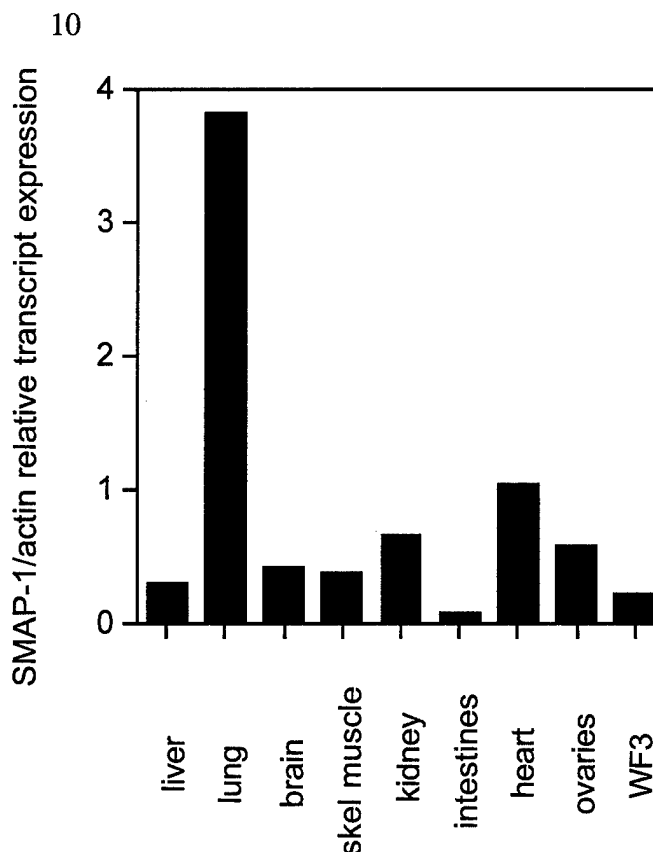
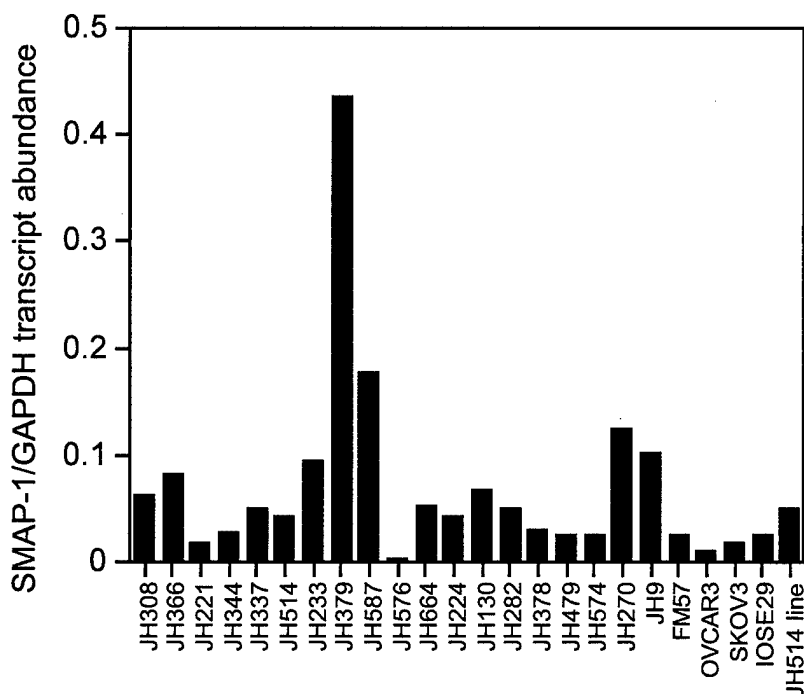


Figure 8. Expression of SMAP-1 transcripts in serous carcinomas and immortalized ovarian surface epithelial cells (IOSE-29). A cDNA preparation from 20 serous carcinoma tissue specimens (JH- and FM-) and three cell lines derived from serous carcinoma was analyzed for GAPDH and SMAP-1 transcripts by Q-PCR. Expression levels of SMAP-1 mRNA are normalized to GAPDH transcript levels.



Key Research Accomplishments

- Developed a new, complementary approach for the identification of tumor-associated antigens
- Identification of 2 new tumor associated antigens for serous carcinoma using the new methodology
- Expression analysis for SMAP-1 transcripts in normal tissue and ovarian tumors
- Finding of over-expression and mislocalization of the SEREX antigen HOXB7 in serous carcinoma

Reportable Outcomes

- Cell line derived from a micropapillary serous carcinoma
- Antisera to HOXB7 and SMAP-1

Conclusions

We have made significant progress in our first year of funding, and have demonstrated that all of the tasks in our Statement of Work are feasible. We have developed a methodology for the identification of ovarian cancer-associated antigens that is complementary to SEREX. While we are generating the cDNA expression library for SEREX screening, we have used this alternate immunoprecipitation and mass spectrometry-based approach to identify two new ovarian cancer associated antigens. Almost nothing is known about the function of either antigen. For one of these new antigens, SMAP-1, we have performed a survey of transcript expression and have developed a specific antibody reagent. Furthermore, we have built upon our previous work in which HOXB7 was identified by SEREX as an ovarian cancer antigen. We have demonstrated over expression of the HOXB7 protein in ovarian cancer, as compared to normal surface epithelium. This immunohistochemical data supports our previous analysis of HOXB7 transcript expression. We also observed a mislocalization of HOXB7 in ovarian cancer. The significance of this exciting finding is under investigation.

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